# Residual Host Cell DNA Measurement


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## Specific Aims

1) Evaluate the linearity and limits of detection of commercial kits used in identifying residual host cell DNA in purified biomedicines and bioprocess intermediates.
2) Translate the assays to digital PCR platforms.

## Background:

Residual host cellular DNA in biomedicines is considered to be a risk factor due to oncogenic or infectivity potential. The U.S. Food and Drug Administration (FDA) guidance to industry recommends that for parenteral inoculation with drugs derived from low-risk host cell types the threshold for residual host cell DNA be no more than 10 ng per dose[1]. For higher risk host cells (e.g. with tumorigenic phenotypes or other risk factors) residual DNA limits should be more stringent. Residual host cell DNA detection may be performed on bioprocess intermediates to monitor the purification process and in the finished product to evaluate purity.

**Quantitative polymerase chain reaction (qPCR)** methods estimate the amount of DNA in a sample. Fluorescence signal is generated by hydrolysis of a labeled probe which hybridizes to the target sequence during PCR amplification. Signal is directly proportional to the quantity of PCR product at each cycle in the PCR reaction. Fluorescence signal is compared to a dilution series of a standard of known DNA concentration to estimate starting DNA quantity in the analyte sample. This method **requires a calibrator curve** to be generated with well characterized quantitative standards.

**Digital PCR (dPCR)** methods use the same fluorescence detection principle as qPCR. However, the PCR reaction is partitioned into hundreds (or thousands) of individual PCR reactions. Partitions which contain a DNA template molecule will generate a fluorescent signal. These positive reactions can be counted to create an estimate of how many DNA molecules are in the sample being analyzed. Absolute DNA copy number is estimated by correlating the number of positive partitions to the volume of sample added to the PCR reaction. This method **does not require an external calibrant**.

## Materials and Methods

**Chinese Hamster Ovary (CHO) cells** were obtained from the NIST Chemical Reference Data Group (645.04) in the Mass Spectrometry Data Center courtesy of Dr. Stephen Stein and Yuxue Liang.

**NIST Candidate Reference Material monoclonal antibody (mAb)** samples were obtained from the Bioanalytical Science Group (645.08) courtesy of John Schiel. Two concentrations of mAb were obtained: 100 mg/mL and 10 mg/mL (Lot #3F1B).

**DNA Extraction:** Cells from either 1 µL or 5 µL of provided CHO cell sample were diluted to a total volume of 200 µL in PBS buffer. DNA was extracted using the **Qiagen DNeasy Blood & Tissue Kit** following the protocol for DNA purification from blood or bodily fluids. A second round of three DNA extractions from 5 µL aliquots was performed as above and the extracts were combined to make a large quantity of DNA for further experiments. This sample is referred to as "CHO Pool".

DNA extraction was performed with NIST mAb samples using the **Applied Biosystems PrepSeq Residual DNA Sample Preparation Kit** on three replicate extractions using 100 µL of sample or control. A quantity of 50 pg of control mouse DNA was spiked into parallel DNA extractions in order to estimate DNA extraction efficiency. Three extraction reagent blanks were run as negative controls.

**Quantitation:** CHO DNA quantity was estimated first on a Life Technologies **Qubit** fluorimeter using the **Qubit dsDNA HS Assay Kit**, then using two qPCR kits: the **Qiagen Certal CHO Detection Kit** and the **Applied Biosystems ResDNASEQ Quantitative CHO DNA Kit**. Mouse NS0 DNA quantity was estimated using the **Applied Biosystems ResDNASEQ Quantitative NS0 DNA Kit.** For the Qiagen CHO kit, a series of 10-fold dilutions of DNA extracted from 5 µL of CHO cells was made as a standard curve. The Qiagen kit does not contain a quantitative standard for estimation of unknowns. For the Applied Biosystems CHO and NS0 kits, standard curves were made with each kit's respective quantitative DNA standard using 10-fold dilutions spanning a concentration range of 0.3 ng/µL to 3 fg/µL per manufacturer's recommendations. Two additional dilutions were made at 0.3 fg/µL and 0.03 fg/µL for ddPCR with the CHO Kit and qPCR with the NS0 Kit. DNA extracts from CHO cells were diluted 1 to 500 in PCR grade water before quantitative analysis by qPCR. "CHO pool" DNA was diluted in a series of six 10-fold dilutions for dPCR.


Figure 3: (A) The Qiagen Certal Residual DNA Detection Kit, (B) the Applied Biosystems PrepSeq Residual DNA Sample Preparation kit, and (C) the Applied Biosystems ResDNASEQ Quantitative NS0 DNA Kit

The qPCR kits from Qiagen (CHO) and Applied Biosystems (CHO and NS0) were run on the Applied Biosystems 7500 Real-Time PCR System using the FAM channel as the detector and ROX as passive reference. The ResDNASEQ CHO kit was also run with the "CHO Pool" DNA extract on the Fluidigm BioMark digital PCR system using a 48.770 chip as a proof of concept that the kit can run on a digital PCR platform. Three replicates of a 10-fold dilution series of control DNA spanning concentrations of 0.3 ng/µL to 0.03 fg/µL was run on panels 1 through 24. A 10-fold dilution series of the pooled CHO DNA extract was run on panels 25 through 42. Negative controls were run on panels 43 through 48.

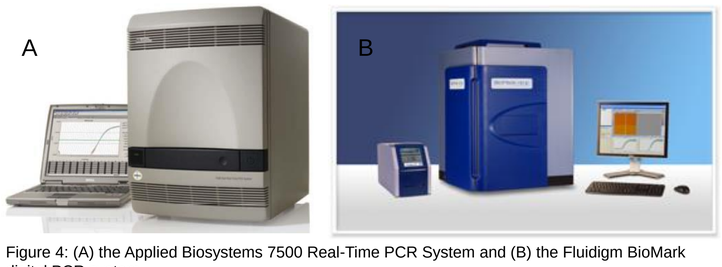


Figure 4: (A) the Applied Biosystems 7500 Real-Time PCR System and (B) the Fluidigm BioMark digital PCR system

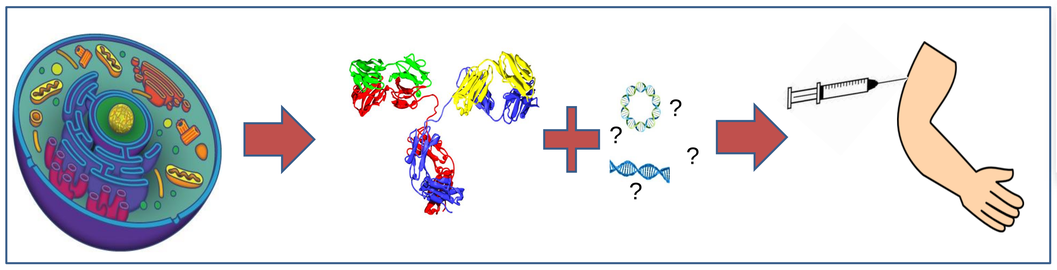

Figure 1: Diagram of a parenterally delivered biopharmaceutical with potential DNA contamination
Image credit: cell - http://biologypop.com/the-evolution-of-the-cells/, viral DNA: http://www.scienceclarified.com/scitech/Genetics/Genes-and-DNA.html

## Experiment 1: Results

Extract DNA: CHO cells; Qiagen DNeasy Kit
Quantitate DNA: Qiagen Certal Residual CHO DNA Detection Kit; ABI 7500 Real-Time PCR System

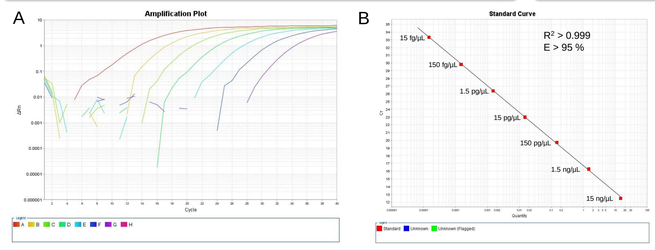


Figure 5: Results of the standard curve from the Qiagen Certal CHO DNA Detection Kit (A) amplification plot and (B) linear regression plot

For the Qiagen CHO kit, the standard curve was linear across a concentration range of approximately 15 ng/µL to 15 fg/µL with $R^2 > 0.999$ and PCR efficiency > 95 %. Quantitation of unknowns was not exact due to lack of a quantitative standard supplied with the kit.

CHO DNA was detected in all dilutions of unknowns spanning approximately 6 ng/µL to 6 fg/µL.

## Experiment 2: Results

Extract DNA: CHO DNA extract (from experiment 1)
Quantitate DNA: Applied Biosystems ResDNASEQ Quantitative CHO DNA Kit; ABI 7500 Real-Time PCR System

Table 1: Concentration estimates from the Invitrogen Qubit dsDNA HS Assay Kit and the Applied Biosystems ResDNASEQ CHO Quantitative DNA Kit on qPCR and dPCR

| Sample Name | Qubit concentration (ng/µL) | qPCR concentration (ng/µL) | dPCR concentration (ng/µL) |
|---|---|---|---|
| CHO 1 µL | 6.3 | 6.0 | |
| CHO 5 µL | 15.1 | 14.6 | |
| CHO Pool | 7.8 | 7.9 | 7.9 |

For the Applied Biosystems CHO kit on the Applied Biosystems 7500, the standard curve was linear across a concentration range of 0.3 ng/µL to 3 fg/µL with $R^2 > 0.999$ and PCR efficiency > 97 %. CHO DNA extracts were quantitatively analyzed and found to be in agreement with values from the Qubit dsDNA HS Assay (see Table 1).

CHO DNA was successfully detected in the standard curve at all dilutions down to 3 fg/µL.

## Experiment 3: Results

Extract DNA: CHO DNA extract (from experiment 1)
Quantitate DNA: Applied Biosystems ResDNASEQ Quantitative CHO DNA Kit; Fluidigm BioMark Digital PCR System

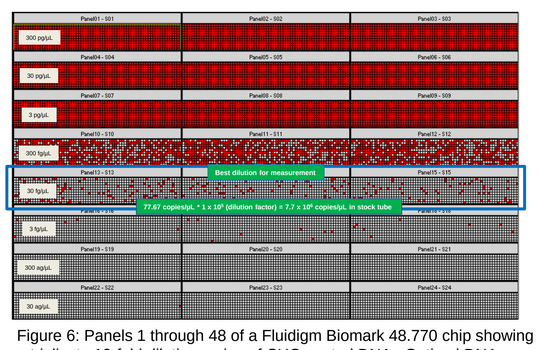


Figure 6: Panels 1 through 48 of a Fluidigm Biomark 48.770 chip showing a triplicate 10-fold dilution series of CHO control DNA. Optimal DNA concentration for measurement is highlighted in the blue box.

When the Applied Biosystems CHO kit was run on the Fluidigm Biomark digital PCR system, the reactions with highest DNA concentration yielded panels that were too saturated to obtain quantitative results. When DNA was diluted to 30 fg/µL (e.g. the 1 x $10^{-5}$ dilution), the number of positive partitions was suitable for copy number determination. Additional standard curve dilutions of 0.3 fg/µL and bellow did not yield enough positive reactions to estimate DNA quantity. Using the 1 x $10^{-5}$ dilution for the unknown, a copy number of 2.06 x $10^7$ was calculated for the CHO Pool sample. Comparing this value to the 1 x $10^{-5}$ standard curve point (which assigns a copy number of 7.77 x $10^6$ to the undiluted DNA standard at 3 ng/µL) yields a concentration estimate of 7.9 ng/µL in the stock "CHO Pool" tube. This number is consistent with Qubit and qPCR estimates (see Table1).

## Experiment 4: Results

Extract DNA: NIST Monoclonal Antibody (mAb); Applied Biosystems PrepSeq Residual DNA Sample Preparation Kit
Quantitate DNA: Applied Biosystems ResDNASEQ Quantitative Mouse NS0 DNA Kit; ABI 7500 Real-Time PCR System

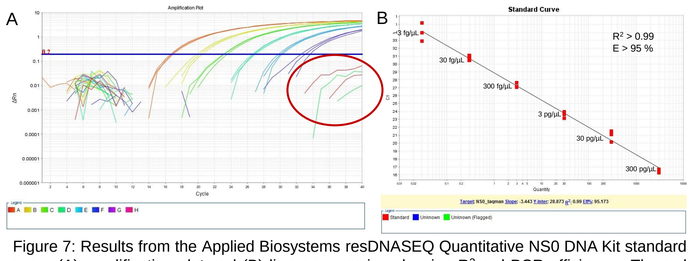


Figure 7: Results from the Applied Biosystems resDNASEQ Quantitative NS0 DNA Kit standard curve (A) amplification plot and (B) linear regression showing $R^2$ and PCR efficiency. The red circle highlights the NIST mAb samples which are below the detection threshold.

For the Applied Biosystems NS0 Kit, the standard curve was linear across all recommended concentrations with $R^2 > 0.99$ and efficiency > 95 %. Two additional standard curve dilutions were added at 0.3 fg/µL and 0.03 fg/µL. No DNA was detected in these extended standard curve points. 10 µL of DNA extract from the NIST mAb were tested with the NS0 kit and no DNA was detected. Reagent controls were also negative.

Extraction positive spike-in controls resulted in concentrations estimated at 0.121 pg/µL for the 10 mg/mL mAb preparation and 0.017 pg/µL for the 100 mg/mL mAb preparation. These samples have a theoretical concentration of 0.5 pg/µL if 100 % efficiency of extraction was obtained. Extraction efficiency is calculated to be 24.1 % for the 10 mg/mL mAb sample and 3.4 % for the 100 mg/mL sample.

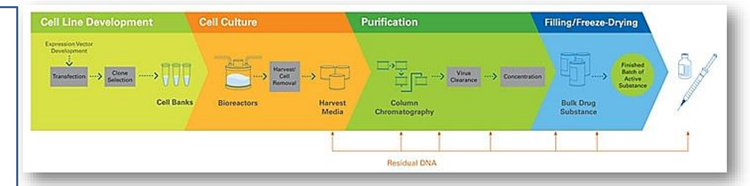


Figure 2: Stages in the biomanufacturing process with residual DNA testing points shown
Image credit: http://www.lifetechnologies.com/us/en/home/life-science/bioproduction/contaminant-and-impurity-testing/host-cell-residual-dna-quantitation.html

## qPCR Calculations:

Coefficient of determination ($R^2$) is used to evaluate the linearity of PCR response across all standard curve dilutions. It is given by the equation :

$$R^2 = 1 - \frac{SS_{res}}{SS_{tot}}$$

Where $SS_{res}$ and $SS_{tot}$ are the residual sum of squares and total sum of squares, respectively.

PCR amplification efficiency (E), a measure of whether the PCR reaction is truly doubling during each cycle, is given by the equation:

$$E = 10^{-1/slope}$$

where slope is determined by linear regression of the standard curve. Efficiency is expressed as a percentage, given by the equation:

$$\%\ Efficiency = (E - 1)\ x\ 100\ \%$$

To calculate DNA extraction efficiency, DNA concentration of spike-in controls was calculated and divided by known starting DNA concentration.

## dPCR Calculations

Using Poisson statistics an estimation of number of copies can be determined. PCR partition volume is given by manufacturer.

$$\textit{Concentration (copies per microliter)} = \frac{ln\left(\frac{\textit{total number of wells}}{\textit{total number of negative wells}}\right)}{\textit{volume of all PCR reactions (microliters)}}$$

## Discussion:

Commercial kits for CHO and mouse NS0 residual DNA detection and quantitation rely on a multi-copy target sequence in the genomes of the host cells. Cell cultures may be subject to genetic drift which could increase or decrease copy numbers of these targets. For this reason, Qiagen does not include a quantitative standard in their DNA detection kit, but rather they recommend that a DNA calibrant be prepared from the same cell line that was used in the biomanufacturing process.

DNA was successfully detected at all dilution points in the recommended concentration range of 0.3 ng/µL to 3 fg/µL. Further dilution resulted in failure to detect the template DNA when using dPCR for CHO and qPCR for mouse NS0. The use of dPCR versus qPCR did not appear to have an effect on lower limits of DNA detection, however more experiments may be required to evaluate quantitative accuracy at extremely low concentrations of DNA. Both the CHO and NS0 kits are fit for the purpose of evaluating DNA concentrations in the concentration range suggested by the FDA guidance for drug purity.

Quantitative PCR has a wide dynamic range of detection. When using digital PCR, samples with unknown composition must be serially diluted across a wide concentration range in order to arrive at a suitable dilution for evaluation of DNA concentration. This may increase the cost of residual DNA detection and quantitation. However, this method may confer tolerance to PCR inhibitors due to the dilution of the starting sample and partitioning of small quantities of template molecules.

Extraction efficiency was found to be lower when starting with a sample containing high concentrations of protein (mAb). The kit manufacturer recommends dilution of highly concentrated bioprocess intermediates prior to DNA extraction.

Sources of error in measurement may include: variation in copy number between the analyte and calibrator when using absolute quantitation in qPCR, mis-assignment of DNA concentration value of the quantitative standard, pipetting error when making the qPCR standard curve resulting in $R^2$ values below 0.99, and in dPCR: the volume of the partition may differ from the nominal volume used by the software to calculate concentration, over- or under-loading of the reaction with template DNA causing deviation from the desired Poisson distribution of template molecules, and in both qPCR and dPCR: under-replication (at least three replicates are needed to accurately assess the variation in the measurement).

## Future Directions:

**Droplet digital PCR (ddPCR):** this method partitions PCR reactions by creating an emulsion of PCR reagents in oil. Broader dynamic range may be obtained through creation of larger numbers of partitions. We plan to expand testing to Bio-Rad QX200 and Rain Dance Raindrop ddPCR platforms.

**Direct PCR:** evaluation of residual DNA may be possible via PCR without first extracting DNA. Sensitivity of the qPCR, and dPCR methods may allow for direct detection simply by diluting the sample beyond the point of any PCR inhibition. Droplet digital PCR may also be amenable to direct PCR due to the possibility of partitioning DNA molecules separately from inhibitory constituents.

**Evaluate commercial reference materials:** U.S. Pharmacopeia (USP) has contacted NIST regarding DNA-based reference standards which it intends to make available for commercial sale. NIST and USP may collaborate on the characterization of a reference material for residual CHO DNA detection. The collaboration would entail evaluation of copy number and concentration using several orthogonal approaches, including qPCR and digital PCR.

**Next generation sequencing:** detection of any adventitious agent's DNA (regardless of species) is possible by using NGS methods. The sensitivity of this method has yet to be established.

## References:


[1] Yang, H. (2013, March-April). Establishing Acceptable Limits of Residual DNA. *PDA J Pharm Sci Tech,* 155-163.